Rainbow trout (Oncorhynchus mykiss) urea cycle and polyamine synthesis gene families show dynamic expression responses to inflammation

Rainbow trout (*Oncorhynchus mykiss*) urea cycle and polyamine synthesis gene families show dynamic expression responses to inflammation

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Abstract

The urea cycle is an endogenous source of arginine that also supports removal of nitrogenous waste following protein metabolism. This cycle is considered inefficient in salmonids, where only 10-15 percent of nitrogenous waste is excreted as urea. In rainbow trout, arginine is an essential amino acid that has attracted attention due to its many functional roles. These roles include the regulation of protein deposition, immune responses and polyamine synthesis; the latter is directly linked to the urea cycle and involved in tissue repair. The key enzymes used in the urea cycle, namely arginase, ornithine transcarbamylase, argininosuccinate synthase and argininosuccinate lyase, in addition to two rate limiting enzymes required for polyamine synthesis (ornithine decarboxylase and s-adenosylmethionine decarboxylase) are poorly studied in fishes, and their responses to inflammation remain unknown. To address this knowledge gap, we characterised these gene families using phylogenetics and comparative genomics, investigated their mRNA distribution among a panel of tissues and established their transcriptional responses to an acute inflammatory response caused by bacterial infection in liver and muscle. Gene duplicates (paralogues) were identified for arginase (*ARG1a*, *1b*, *2a* and *2b*), ornithine decarboxylase (*ODC1* and *2*) and s-adenosylmethionine decarboxylase (*SAMdc1* and *2*), including paralogues retained from an ancestral salmonid-specific whole genome duplication. *ARG2a and 2b* were highly upregulated following bacterial infection in liver, whereas *ARG1b* was downregulated, while both paralogues of *SAMdc* and *ODC* were upregulated in both liver and muscle. Overall, these findings improve our understanding of the molecules supporting the urea cycle and polyamine synthesis in fish, highlighting major changes in the regulation of these systems during inflammation.

Key words: Urea cycle, polyamine, salmonids, genome duplication, immune, arginase

1. Introduction

The ornithine-urea cycle, first discovered by Krebs and Henseleit [1], is central to the metabolism of arginine and the excretion of nitrogenous waste. In fish, most nitrogenous waste is excreted as ammonia through the gills, with <10% excreted as urea in rainbow trout [2]. There are four main enzymes directly involved in the urea cycle, arginase, ornithine transcarbamylase, argininosuccinate synthase and argininosuccinate lyase, which metabolise and recycle arginine, ornithine, citrulline and argininosuccinate [3]. Arginine is a versatile amino acid with functional roles including the modulation of protein deposition, production of ornithine for polyamine synthesis, regulation of immune responses through nitric oxide (NO) production, and removal of nitrogenous waste [4]. Arginine also stimulates the release of insulin, glucagon and growth hormone in fishes, which may regulate metabolism and growth [5].

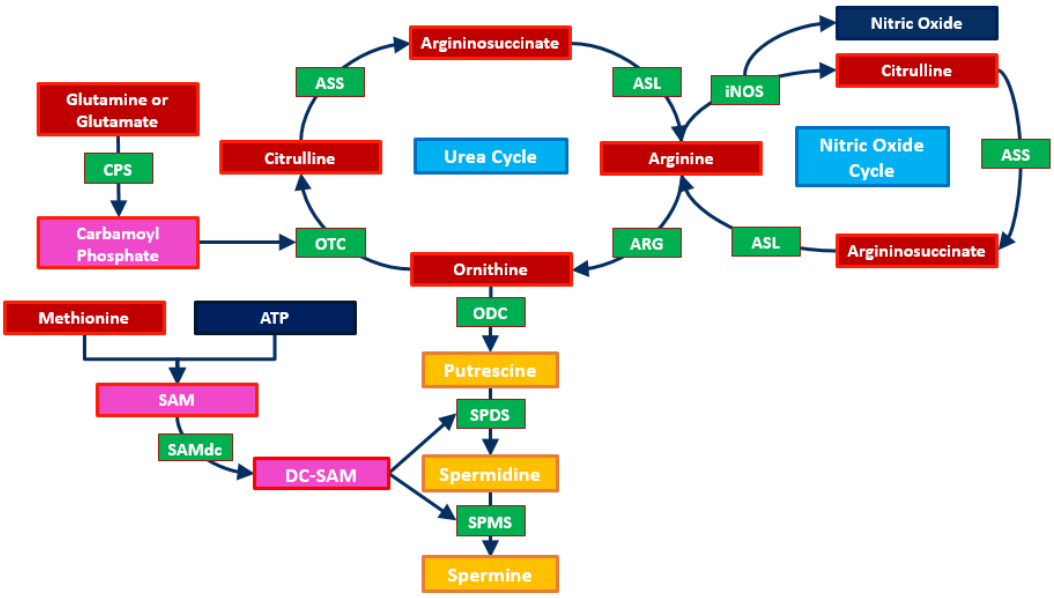

Figure 1. Pathway diagram of urea cycle, nitric oxide cycle and polyamine synthesis. Amino acids are coloured in red boxes, enzymes in green, polyamines in orange, co-substrates in pink and molecules as dark blue. Enzyme acronyms are as follows: ARG, arginase; OTC, ornithine transcarbamylase; ASS, argininosuccinate synthase; ASL, argininosuccinate synthase; CPS, carbamoyl phosphate synthase; iNOS, nitric oxide synthase; SAMdc, S-adenylosylmethionine decarboxylase; ODC, ornithine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase. Co-substrate acronyms are as follows: SAM, s-adenosylmethionine; DC-SAM, decarboxylated s-adenosylmethionine.

During the urea cycle (Fig. 1), arginine is converted to ornithine by the arginase (*ARG*) enzymes, resulting in urea as the by-product. Ornithine can then be converted into citrulline by ornithine transcarbamylase (*OTC*), used in proline synthesis by the action of ornithine aminotransferase, or for polyamine synthesis, where ornithine decarboxylase (*ODC*) converts ornithine into the polyamine putrescine [6]. Recycling citrulline back into arginine is

a two-step process that involves two further enzymes (*ASS* and *ASL*), as well as argininosuccinate as an intermediate [7]. Citrulline and argininosuccinate also form part of the NO cycle (Fig. 1), as citrulline is generated as a by-product following conversion of arginine to NO through the action of the NO synthase enzymes [8, 9].

An important set of molecules derived from the urea cycle are the polyamines, containing two or more amine groups (-NH_2). The diverse functions of polyamines include regulation of protein synthesis [10], modulation of ion channels [11] and DNA and RNA binding [12]. They are also crucial for cellular proliferation and inflammatory responses [13], acting as anti-oxidants and offering cellular protection through inhibition of inflammatory mediators [14]. Polyamines are derived from ornithine by the activity of *ODC* and s-adenylosylmethionine decarboxylase (*SAMdc*), with the simplest polyamine being putrescine, which can be further processed to spermidine and spermine (Fig. 1) [7]. During the immune response, high polyamine levels are found in rapidly proliferating cells and tissues [15, 16] supporting wound and tissue healing following infection or injury [6, 17].

The role of arginine and urea cycle products is attracting attention due to their roles in mediating immune functions. Inflammatory responses are associated with polarising T_H1 cytokines including IFN-γ- or TNFα-activating M1 macrophages ('kill' macrophages), whereas anti-inflammatory processes activate M2 macrophages ('healing' macrophages) associated with T_H2 cytokines including interleukin 4 and 10 (*IL-4, IL-10*) [18]. M1 macrophages metabolise arginine into NO via inducible nitric oxide synthase (*iNOS*), resulting in a macrophage population with increased microbicidal activity [19]. On the other hand, anti-inflammatory responses and healing is associated with M2 cells, where arginine is converted to ornithine and subsequently metabolised to polyamines through *ODC* [20]. As M1 and M2 macrophages compete for arginine, the expression of either *iNOS* or arginase has a reciprocal regulatory effect [21]. Arginine is the sole precursor of NO and supplementation is known to increase NO synthesis in mammals [22].

Arginine is a functional feed that can modulate health and performance parameters in farmed fish. For example, arginine supplementation may enhance growth in Atlantic salmon during the transition from fresh to seawater [23], improve immune status in carp [24] and when combined with glutamine, enhance growth and feeding efficiency in Nile tilapia [25]. Despite such recent interest, little is known about the gene families encoding the enzymes involved in arginine metabolism and their response to disease and inflammation in fishes.

The first objective of this study was to identify and characterise the genes encoding the main urea cycle enzymes and the two rate limiting enzymes in polyamine synthesis in rainbow trout, including any paralogues retained during salmonid evolutionary history. The second

objective was to establish the mRNA expression of these genes in rainbow trout, performed in a panel of tissues under control conditions, and following a bacterial pathogen challenge in liver and muscle. The resultant data implies an important role for arginine in both inflammation and tissue repair.

2. Materials and Methods

2.1. Animal work

All procedures described hereafter were carried out in compliance with the Animals (Scientific Procedures) Act 1986 under UK Home Office license PPL number 70/8071 and approved by the ethics committee at the University of Aberdeen. Juvenile rainbow trout were purchased from College Mill Trout Farm (Perthshire, U.K.). The fish were kept at the University of Aberdeen aquarium facility (School of Biological Sciences) in 400 L tanks at a stocking density of <20 kg/m^3. Tanks were supplied with recirculating freshwater with a flow rate of 1.5 L/s. Fish were kept at a temperature of 14 ± 1^{o}C and a photoperiod of 12:12 light:dark. A computerised control system was used to monitor pH, ammonia concentration and oxygen levels. Fish were fed *ad libitum* daily with commercial pellets.

To assess candidate gene expression responses across tissues, n = 4 adult rainbow trout (499 ± 54 g mean ± SEM) were used to sample a standard panel of tissues; gill, distal intestine, heart, head kidney, liver, fast-twitch skeletal muscle and spleen (within 5 minutes of death). Tissues were stored in 1.5 ml RNA later at 4^{o}C for 24 h followed by long term storage at -80^{o}C.

For the bacterial immunological stimulation, fish (as described above) were anaesthetised by immersion in 2-phenoxyethanol and then injected intraperitoneally (ip) with either phosphate buffered saline (PBS) (0.5 ml/fish) or the pathogenic Hooke strain of the live Gram-negative bacterium *Aeromonas salmonicida* (AS) (1.6 x 10^6 ml^{-1} cells, 0.5 ml/fish). After ip injection, the fish were maintained in 400 L tanks in the University of Aberdeen's freshwater challenge facility for 48 h. After 48 h, n=10 fish from both PBS and AS groups were randomly sampled and killed as previously described and both liver and fast-twitch skeletal muscle tissue sampled and stored in RNA later as described above.

2.2. Sequence, phylogenetic and genomic analysis

Putative protein-coding nucleotide sequences (cds) for candidate genes were originally obtained from the rainbow trout genome hosted at https://www.genoscope.cns.fr/trout/ (NCBI

accession: GCA_900005705.1) [26]. This was achieved using BLASTn searches with human orthologues downloaded from NCBI as the query: *ODC* (AH002917.2), *SAMdc* (NM_001634.5), *ARG1* (NM_001244438.1), *ARG2* (NM_001172.3), *OTC* (NM_0005315), *ASS* (AH002610.2) and *ASL* (M14218.1). These sequences were also consistent when compared to the rainbow trout genome deposited on NCBI (GCF_002163505.1). For additional phylogenetic analysis, further vertebrate (coding) CDS sequences were retrieved from NCBI and/or Ensembl [27] databases. Protein sequences from Atlantic salmon, rainbow trout, northern pike, zebrafish, spotted gar, chicken, mouse and human were retrieved and accession numbers for all protein sequences are displayed in Supplementary Table 1. MatGat [28] was used to predict amino acid identity/similarity between all vertebrate proteins for each gene. Protein sequences were aligned using ClustalW in the MEGA7 software [29]. A phylogenetic tree was constructed using the Jones-Taylor-Thornton model in the maximum likelihood method in MEGA 7, bootstrapped 500 times.

Intron-exon structure and gene synteny analysis was carried out for all candidate gene families. To determine the genomic neighbourhood around candidate genes and the conservation of gene order across the same species mentioned earlier, genes were manually examined in NCBIs genomic region browser. Intron-exon structures were determined from the same databases used to retrieve cds sequences.

2.3. Primer design for quantitative PCR (qPCR)

Due to the duplicated nature of salmonid genomes, care was taken to design paralogue-specific primers for genes with more than one copy within the genome (details of all primers in Table 1). Nucleotide mRNA sequences were therefore aligned with ClustalOmega [30] in order to compare paralogues and identify distinguishing regions of sequence. To avoid amplification of genomic DNA, primers were designed to span an intron-exon junction or were placed in different exons. Primers were also designed to have an annealing temperature of ~64^{o}C judged from OligoCalc [31] and a product length of between 100 and 330 bps. Prior to qPCR analysis (section 2.5), confirmation of PCR products generated using the paralogue-specific primers was carried out by cloning and sequencing. For confirmatory sequencing the PCR products were ligated into pGEM-T easy cloning vector (Promega) and then transformed into competent *Escherichia coli* cells (JM109). Plasmid DNA was isolated by Qiagen mini prep kits as described by manufacturer. A minimum of 5 clones per paralogue were sent for Sanger sequencing, carried out by Eurofins.

Gene	Sense	Primer 5'-3'	Product size	Annealing temperature	Accession
EF-1α[1]	Forward	CAAGGATATCCGTCGTGGCA	327	64	NM_001124339.1
	Reverse	ACAGCGAAACGACCAAGAGG			
β–actin[2]	Forward	ATGGAAGATGAAATCGCCCC	260	64	XM_021595779.1
	Reverse	TGCCAGATCTTCTCCATGTCG			
HPRT[3]	Forward	CCGCCTCAAGAGCTAGTGTAAT	237	64	XM_021583468.1
	Reverse	GTCTGGAACCTCAAACCCTATG			
RPS29[1]	Forward	GGGTCATCAGCAGCTCTATTGG	167	64	XM_021612450.1
	Reverse	AGTCCAGCTTAACAAAGCCGATG			
SAA	Forward	TATGATGCTGCCAGGAGAGGAC	137	64	NM_001124436.1
	Reverse	CGTCCCCAGTGGTTAGCCTT			
HAMP	Forward	AGGAGGTTGGAAGCATTGACAG	101	64	XM_021595153.1
	Reverse	GTGGCTCTGACGCTTGAACCT			
ODC1	Forward	CGTGTGCCAGCTCAGTGTC	179	64	XM_021574142.1
	Reverse	CCATGTCAAAGACACAGCGG			
ODC2	Forward	TGGTGCCACCCTGAAGGCC	128	64	XM_021585068.1
	Reverse	AGATGGCCTGGCTGTAGGTG			
SAMdc1	Forward	GCAAGGACAAGCTAATTAAG	185	64	XM_021600286.1
	Reverse	AACCTTGGGATGGTACGGAG			
SAMdc2	Forward	AACTCACGATGGAAGCGAAC	121	64	XM_021611778.1
	Reverse	AACCTTGGGATGGTACGGAG			
ARG 1A	Forward	AGCACCATATCCTGACGTTG	147	64	XM_021564871.1
	Reverse	CATCGATGTCATAGCTCAGG			
ARG 1B	Forward	GGTGGATCGCCTTGGAATCG	179	64	KX998966.1
	Reverse	CTGTGATGTAGATTCCCTCC			
ARG 2A	Forward	TCCAGAGAGTCATGGAAGTCACTTTCC	198	64	KX998967.1
	Reverse	CCATCACTGACAACAACCCTGTGTT			
ARG 2B	Forward	CTTGTTGAGGTCAACCCAGC	163	64	KX998968.1
	Reverse	GTCGAAGCTGTTCCGTGTCG			
OTC	Forward	CACAGCCAGGGTTCTCTCTG	116	64	XM_021597830.1
	Reverse	CAGACAGGCCGTTGATGATG			
ASS	Forward	TGAGATTGGAGGGAGGCATG	172	64	XM_021590913.1
	Reverse	GCCCTGTTTGATCCTCCTGA			
ASL	Forward	ACGCTCTCCAACTCATCACA	129	64	XM_021563243.1
	Reverse	ACCGCATGACTCAGAATCCA			

173 *Table 1. Rainbow trout primer sequences used for qPCR with NCBI accession numbers*
174 References [1][32], [2] [33], [3] [34]

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2.4. RNA extraction and reverse transcription

Total RNA was extracted from 100mg of tissue homogenised in 1ml of TRI Reagent (Sigma-Aldrich) following the manufacturer's instructions. The concentration and purity of RNA was estimated using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). The integrity of RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). First-strand cDNA was synthesized from 1 μg total RNA using a QuantiTech Reverse Transcription kit (QIAGEN), with an integrated genomic DNA elimination step, as per the manufacturer's guidelines in a total volume of 20 μl. First strand cDNA samples were diluted 20-fold (working stock) with RNase/ DNase free water (Sigma-Aldrich) and stored at -20°C until use.

2.5. Quantitative gene expression analysis

qPCR analyses were performed with SYBR Green I dye chemistry using an Mx3005P System (Agilent Technologies). All assays were carried out in duplicate within 96 well plates using 15 µl reactions containing 5 µl of the 1:20-diluted cDNA (corresponding to 2.5 ng of reverse-transcribed total RNA), 500 nM sense/antisense primers and 7.5 µl Brilliant III Ultra-Fast SYBR Green (Agilent Technologies). The PCR cycling conditions were 1 cycle of 95°C for 3 minutes, followed by 40 cycles of 95°C for 20 seconds then 64°C for 20 seconds (two step PCR). Melting curve analysis (thermal gradient from 55°C to 95°C) was used to confirm the amplification of a single product. Each plate also included no-template controls in duplicate (cDNA replaced with water). Threshold fluorescence was set at 2500 during the linear phase of amplification. The efficiency of each qPCR assay was assessed using LinRegPCR quantitative PCR data analysis program (download: http://LinRegPCR.HFRC.nl) following Ruijter *et al* [35] recommendations. Expression data was then imported and analysed in Genex 5.4.3 (MultiD Analysis). Candidate gene expression was normalised to two reference genes for the tissue distribution (*EF-1α* and *HPRT*) and three genes for the infection study on muscle (*EF-1α, HPRT* and *RPS29*) and liver (*EF-1α, ACTB*, and *HPRT*). *ACTB* was replaced with *RPS29* in muscle due to *ACTB's* instability in muscle determined from the dissociation graph following qPCR. All reference gene primers used in the study are presented in Table 1.

2.6. Statistical analysis

Statistical analysis of qPCR data was performed in R (v3.4.0). A linear model (lm) was first constructed in R and the diagnostic plots (qq plot and residuals versus fitted values) were assessed in order to ensure both normality and equal variance. If data met the assumptions,

the one-way ANOVA results from R's lm function could then be interpreted. If data was not normal, a log transformation was first performed and the diagnostics plots then reassessed. If the data still did not meet the models assumptions following the transformation, a non-parametric test (Kruskal wallis) was then performed on the data.

3. Results

3.1 Comparative analysis of the urea cycle genes

The phylogenetic relationships of all genes were carried out in comparison to a range of representative vertebrate lineages. Two copies of *ARG1*, *ARG2*, *SAMdc* and *ODC* were identified, along with single copies of *OTC*, *ASS* and *ASL*. Gene intron-exon structure, amino acid sequence analysis and synteny were performed on *ODC* as a representative example.

Two *ODC* paralogues were identified in the rainbow trout genome (*ODC1* accession: XM_021574142, Chr19, LOC110498573, *ODC2* accession: XM_021585068.1, Chr25, LOC110505682). The open reading frame for *ODC1* and *ODC2* encoded 457 amino acids (aa) and 456 aa respectively, and a conserved gene structure of 8 exons and 7 introns (Fig. 2) was evident between the paralogues. Across species, exons 2 and 3 were identical lengths for all species examined, while exon 4 was conserved in all teleosts, whereas in the tetrapods an additional intron is present resulting in tetrapod exons 4 and 5 being the same length as teleost exon 4 (Fig. 2). Similarly, within salmonid exon 5, an additional intron can be found in the remaining species analysed (Fig. 2). Exons 6 and 7 in salmonids and corresponding exons in the rest of the vertebrate species are highly conserved with tetrapods having 3 nucleotides less in relation to salmonid exon 6 and all species having identical length in salmonid exon 7 (Fig. 2).

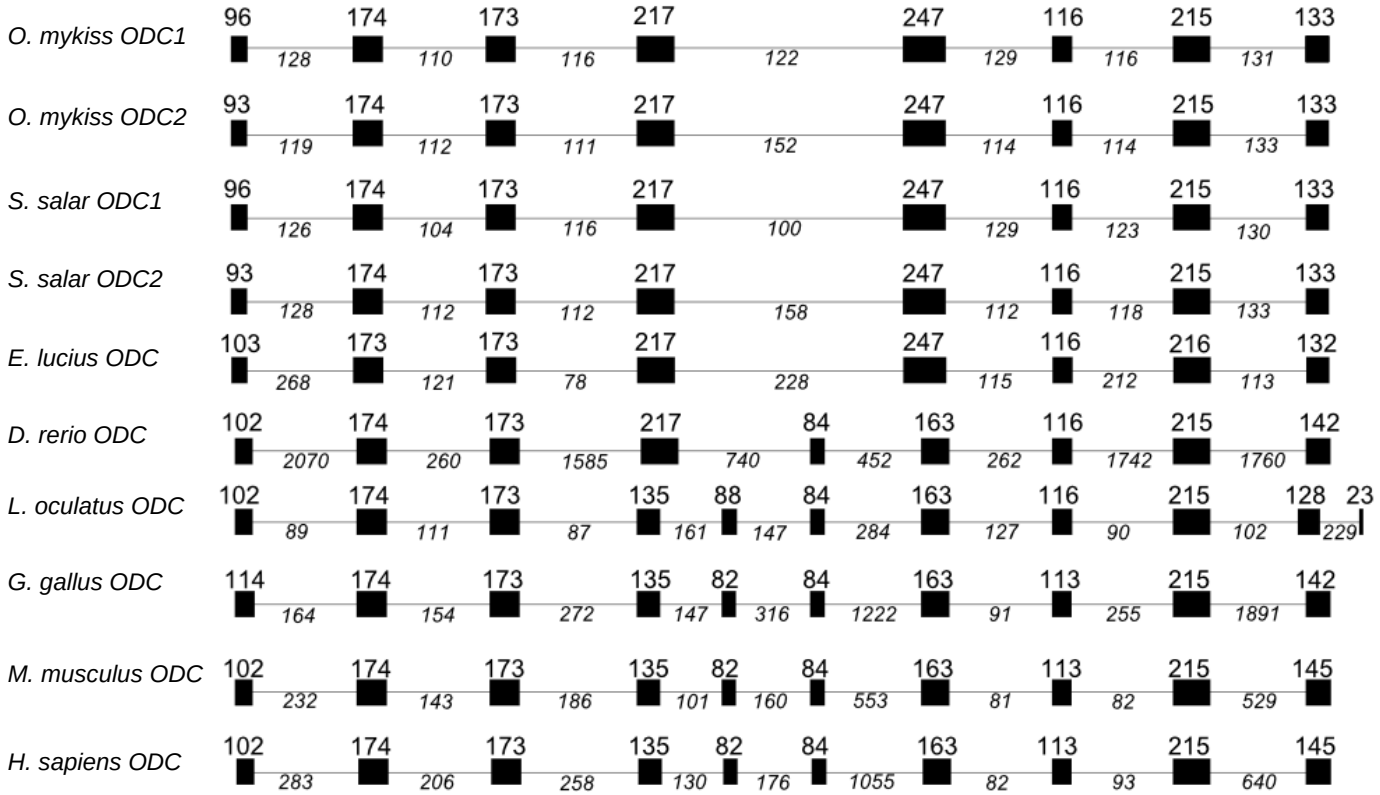


Figure 2. Intron and exon structure of ODC coding regions across vertebrates, 5' and 3' UTRs not shown. Black boxes represent exons and are drawn to scale with nucleotide base pair sizes indicated above. Black lines connecting exons are introns and are not drawn to scale with sizes indicated below the line in italics. The sequences used for the intron exon analysis are as follows: O. mykiss ODC1 (XM_021574142.1), O. mykiss ODC2 (XM_021585068.1), S. salar ODC1 (XM_014192087.1), S. salar ODC2 (XM_014211026.1), E. lucius ODC (XM_010892375.3), D. rerio ODC (ENSDARG00000007377), L. oculatus (ENSLOCT00000020613.1), G. gallus ODC (ENSGALT00000026527.5), M. musculus ODC (ENSMUST00000171737.1), H. sapiens ODC (ENST00000234111.8)

Phylogenetic analysis of the *ODC1* and *2* proteins revealed that each molecule has an orthologue in both Atlantic salmon and rainbow trout that shared ~98% aa identity (Table 2). Within species, both rainbow trout and salmon *ODC1* compared to *ODC2* has 93.4% and 94.7% identity respectively (Table 2). The presence of the genes on distinct chromosomes and the branching of northern pike as a sister group to both *ODC1* and *2* suggests these genes are products of the salmonid specific WGD (ssWGD) (Fig. 3). *ODC1* and *2* are highly conserved with other vertebrate *ODC* orthologues (Table 2), for example sharing ~73% aa level identity each with human.

	O. mykiss ODC1	*S. salar ODC1*	*O. mykiss ODC2*	*S. salar ODC2*	*E. Lucius ODC*	*D. rerio ODC*	*L. oculatus ODC*	*G. gallus ODC*	*M. musculus ODC*	*H. sapiens ODC*
O. mykiss ODC1		98.2	93.2	94.5	92.4	85	77.5	71.4	71.2	72.5
S. salar ODC1	99.3		93.4	94.7	92.8	84.6	77.9	70.8	71	72.3
O. mykiss ODC2	96.1	96.5		98.2	92.6	85.5	79.4	71	72.1	72.5
S. salar ODC2	96.9	97.1	98.9		93.4	85.9	79	71.4	72.1	72.9
E. lucius ODC	95.4	96.1	95.2	95.9		87	79.2	71.7	72.5	72.7
D. rerio ODC	90.7	91.8	92	92	93.1		80.1	73.7	73.9	74.1
L. oculatus ODC	87.6	88	88.2	87.3	87.6	89.7		79.4	76.8	78.3
G. gallus ODC	83.8	83.6	82.5	82.5	82.6	86.4	89.3		81.5	83.9
M. musculus ODC	84.6	85.2	85.2	84.8	84.2	87	88.4	91.2		90.7
H. sapiens ODC	83.9	84.6	83.5	83.7	83.3	85.5	89.5	92.9	94.1	

Identity | Similarity

Table 2. Comparison of amino acid identities and similarities (%) for ODC using the similarity matrix in MatGat 2.02 software. Accession numbers for all proteins can be found on Supplementary Table 1

Phylogenetic analysis further confirmed *ODC1, ODC2* (Fig. 3) and *SAMdc1, SAMdc2* (Supplementary Fig. 1) as products of ssWGD with higher relatedness to their salmonid relative than to their own species duplicate. In the case of the *ARG1* and *ARG2*, these genes are present in all vertebrates and likely diverged before the evolution of vertebrates. However as there are 4 copies of arginase present in salmonids (*ARG1a, ARG1b, ARG2a* and *ARG2b*), it can be seen that the two paralogues for arginase 1 and 2 are likely products of the ssWGD (Supplementary Fig. 2). All other genes characterised in this study (*OTC, ASS, ASL*) have not retained a duplicate copy (Supplementary Figures 3-5).

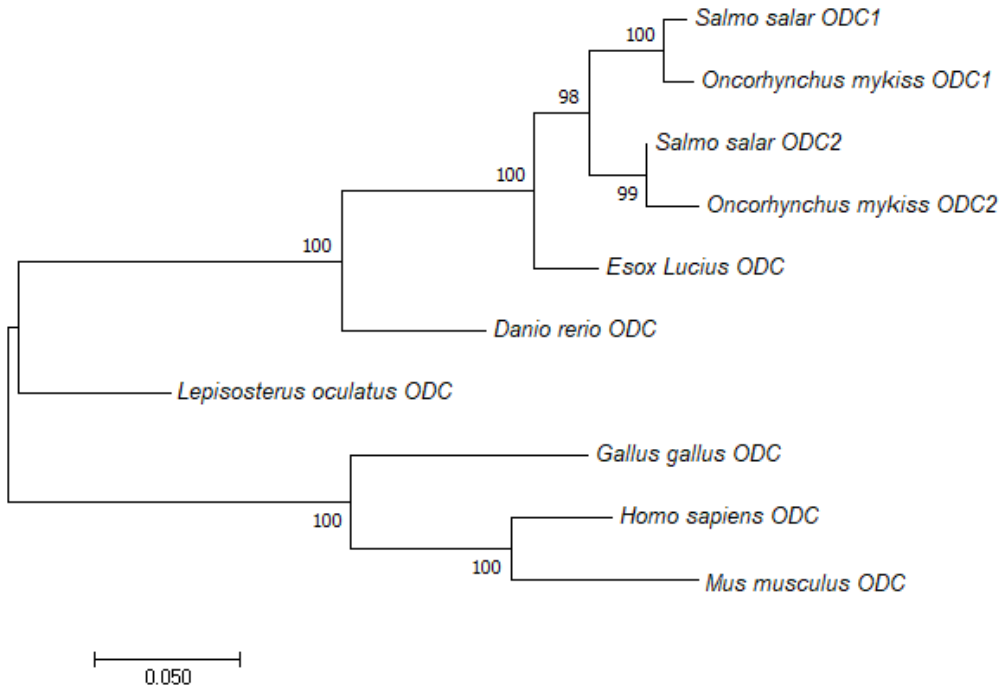


Figure 3. Maximum likelihood phylogenetic tree showing the evolutionary relationship of ODC amino acid sequences in vertebrates. The branch support values were gained by non-parametric bootstrapping (500 replicates). The scale bar represents the number of modelled substitutions per site. Accession numbers from NCBI are as follows: O. mykiss ODC1 (XP_021429817.1), O. mykiss ODC2 (XP_021440743.1), S. salar ODC1 (XP_014047562.1), S. salar ODC2 (XP_014066501.1), E. lucius ODC (XP_010890677.1), L. oculatus ODC (XP_006626107.1), D. rerio ODC (NP_571876.1), G. gallus ODC (NP_001161238.1), M. musculus ODC (NP_038642.2), H. sapiens ODC (AAA59968.1)

Synteny analysis revealed highly conserved gene order in the genomic regions containing *ODC1* and *2*, both in relation to each other and also in comparison to single copy *ODC* orthologues in non-salmonid teleosts and tetrapod vertebrates (Fig. 4). At the 5' of *ODC*, the most proximal neighbouring gene (*NOL10*) was retained in the same location in all vertebrates. We also identified several other annotated genes with high synteny located near *ODC* including *KCNA1* and *ROCK2*. Towards the 3' end of *ODC*, salmonid genes shared similarity of gene order with the northern pike, but with no other vertebrates including zebrafish, suggesting a chromosomal rearrangement prior to the salmonid / pike divergence from other teleosts. As for the genes downstream of *ODC*, we identified some annotated genes in two copies on both the *ODC1* and *2* chromosomes, barring the *YIPF* family of genes. Towards the 3' of rainbow trout *ODC1* there was no *YIPF* gene annotated, but in Atlantic salmon *YIPF* was identified downstream of both *ODC1* and *2*.

Figure 4. Phylogenetic tree and gene synteny of ODC in vertebrates. The tree was constructed using the maximum likelihood method in MEGA7 and bootstrapped 500 times. The syntenically conserved gene blocks are shown in matching colours. The arrows represent transcriptional direction. Gene synteny was compiled from up and downstream locations relative to each species ODC taken from NCBI, ODC protein accession numbers on supplementary table 1, chromosome number and range (from left of the diagram to right) as follows: O. mykiss ODC1 (Chr 19, 34,937,046 > 35,209,567), O. mykiss ODC2 (Chr 25, 63,402,440 > 63,152,107), S. salar ODC1 (Chr ssa01, 29,382,254 > 29,167,678), S. salar ODC2 (Chr ssa09, 27,309,511 > 27,043,274, E. lucius ODC (Chr LG15 , 14,186,061 > 14,321,347), D. rerio ODC (Chr 17, 51,655,239 > 51,757,548), L. oculatus ODC (Chr LG1, 43,496,860 > 43,340,082), G. gallus ODC (Chr 3, 97,175,730 > 96,773,203), M. musculus ODC (Chr 12, 16,894,978 > 17,791,944), H. sapiens ODC (Chr 2, 11,179,759 > 10,427,617).

3.2. Urea cycle and polyamine synthesis gene expression

3.2.1 *Tissue distribution*

The relative mRNA expression levels of the characterized urea cycle (*ARG1a, ARG1b, ARG2a, ARG2b, OTC, ASS and ASL)* and rate limiting enzymes of polyamine synthesis (*ODC1*, *ODC2*, *SAMdc1* and *SAMdc2*) were quantified by qPCR in seven tissues in healthy rainbow trout under control conditions (Fig. 5 for *ODC1* and *2*; all other genes: supplementary figures 6-8). Both *ODC* paralogues were expressed in all tissues examined, with *ODC2* less abundant than *ODC1* in all tissues barring gill (Fig. 5). *ODC1* was most highly expressed in head kidney followed by spleen and gill. *ODC2* had the highest expression in gill followed by head kidney.

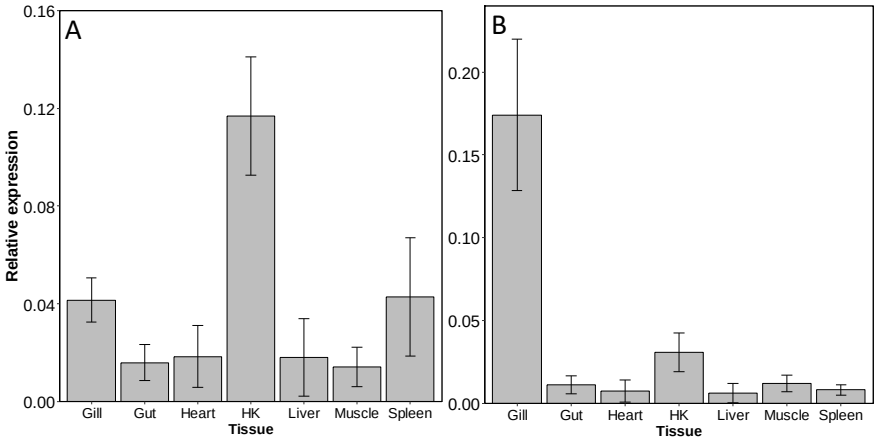


Figure 5. Tissue distribution of ODC1 (A) and ODC2 (B) in rainbow trout. The relative expression in each tissue was normalised with the expression of two housekeeping genes EF-1α and HPRT. Bars represent mean (± SEM), n=4.

The four arginase paralogues showed a more variable expression distribution (Supplementary Fig. 6). *ARG1a* and *ARG1b* both showed highest expression in the liver (Supplementary Fig. 6). *ARG1a* was expressed in all tissues examined, with highest expression in liver and gill. *ARG1b* showed highest expression in liver, with lower expression levels in gill, heart and muscle, and no detectable expression in gut, head kidney or spleen expression. *ARG2a* and *ARG2b* were expressed in all tissues, both showing highest expression in the muscle and lowest expression in liver.

The single copy urea cycle genes *OTC*, *ASS* and *ASL* were expressed in all the tissues examined, with *OTC* and *ASL* having highest expression in muscle and *ASS* in gill (Supplementary Fig. 7). *OTC* was expressed at a very low level in the liver (Supplementary Fig. 7). The two *SAMdc* paralogues were expressed in all tissues, with both genes having highest expression in heart and muscle (Supplementary Fig. 8).

3.2.2. Modulation of mRNA expression following bacterial infection

Expression of the urea cycle enzymes and polyamine synthesis genes was examined in adult rainbow trout liver and muscle tissue sampled following an experimental infection with a pathogenic strain of the bacterium *Aeromonas salmonicida* (AS). To confirm the fish were undergoing an inflammatory response to the infection, the expression of two marker genes for the acute phase response (APR), serum amyloid A (*SAA*) and hepcidin (*HAMP*), were examined in control and AS-infected fish. Both genes showed highly significant upregulation in infected fish (Fig. 6) indicating a strong immune response.

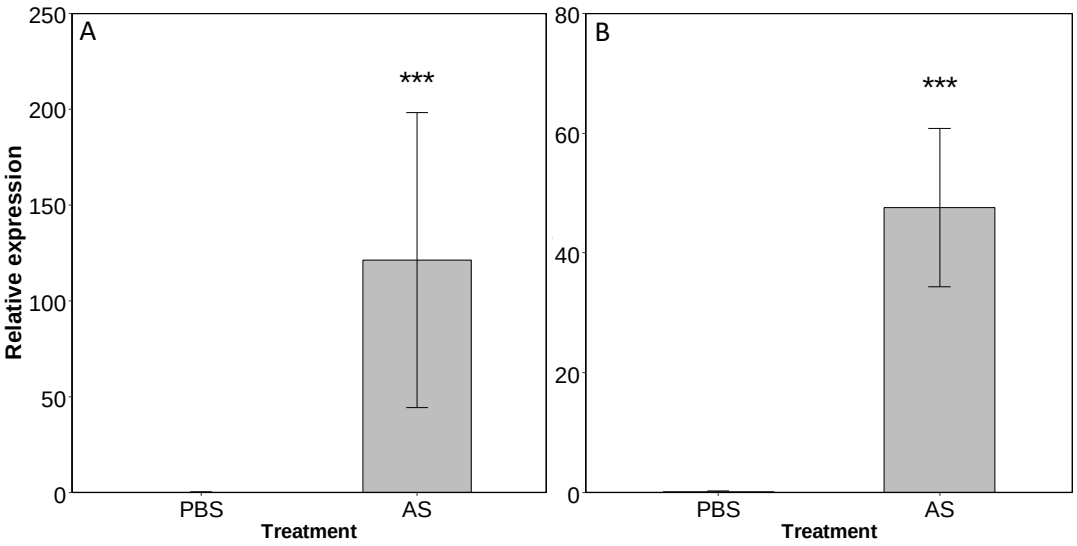


*Figure 6. Relative expression of rainbow trout SAA (A) and HAMP (B) in liver tissue following a bacterial infection. Fish were injected intraperitoneally with either phosphate buffered saline (PBS) or Aeromonas salmonicda (AS). SAA and HAMP expression was normalised to housekeeping genes EF-1α, ACTB and HPRT. Linear model in R was used for analysis of both genes. Bars represent mean (± SEM), n=10, *** = p < 0.001*

Following AS infection both *ODC* paralogues were significantly upregulated in the liver (Fig. 7). In muscle, *ODC1* and *2* expression did not change following infection (Fig. 7). Both *SAMdc1* and *2* were significantly upregulated in liver but not muscle (Fig. 7). *ARG1a* was significantly downregulated in liver and unchanged in muscle whereas the opposite pattern was observed for *ARG1b* (Fig. 8). *ARG2a* and *ARG2b* expression was significantly increased in liver, with *ARG2b* also significantly increased in muscle but not *ARG2a* (Fig. 8). Both *OTC* and *ASL's* expression was significantly increased in muscle, but not liver (Fig. 9), whereas *ASL* expression increased significantly in infected liver but not in muscle (Fig. 9).

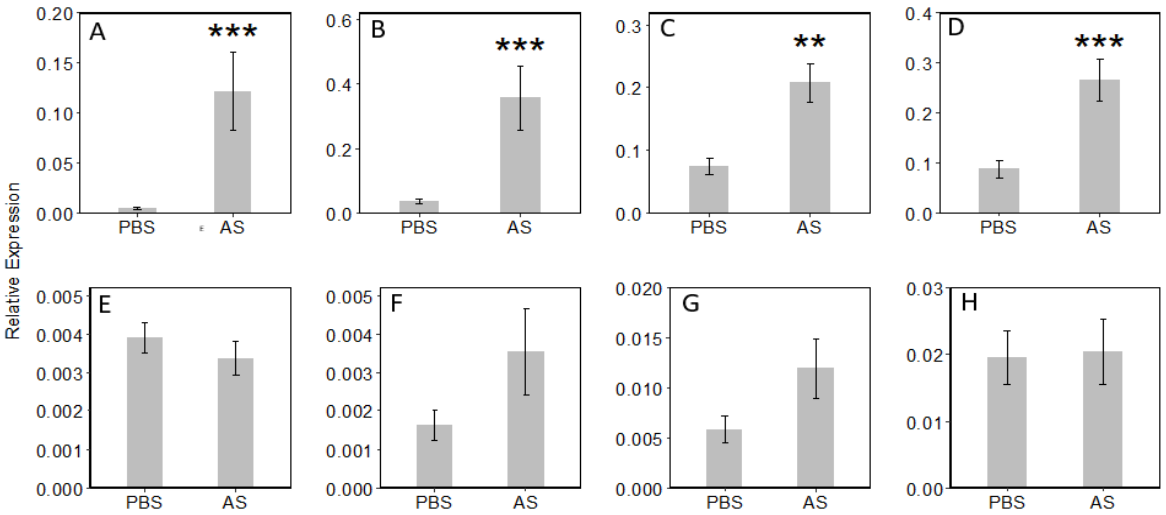


*Figure 7. Relative expression of the rate limiting enzymes in polyamine synthesis, SAMdc and ODC, in rainbow trout muscle and liver tissue following a bacterial infection. Fish were injected intraperitoneally with either phosphate buffered saline (PBS) or Aeromonas salmonicda (AS). The top row of graphs show gene in liver tissue: ODC1 (A), ODC2 (B), SAMdc1 (C), SAMdc2 (D), expression was normalised to housekeeping genes EF-1α, ACTB and HPRT. The bottom row of graphs show gene expression in muscle tissue: ODC1 (E), ODC2 (F), SAMdc1 (G), SAMdc2 (H), expression was normalised to housekeeping genes EF-1α, RPS29 and HPRT. Bars represent mean (± SEM), n=10, * = p < 0.05, ** = p < 0.01, *** = p < 0.001*

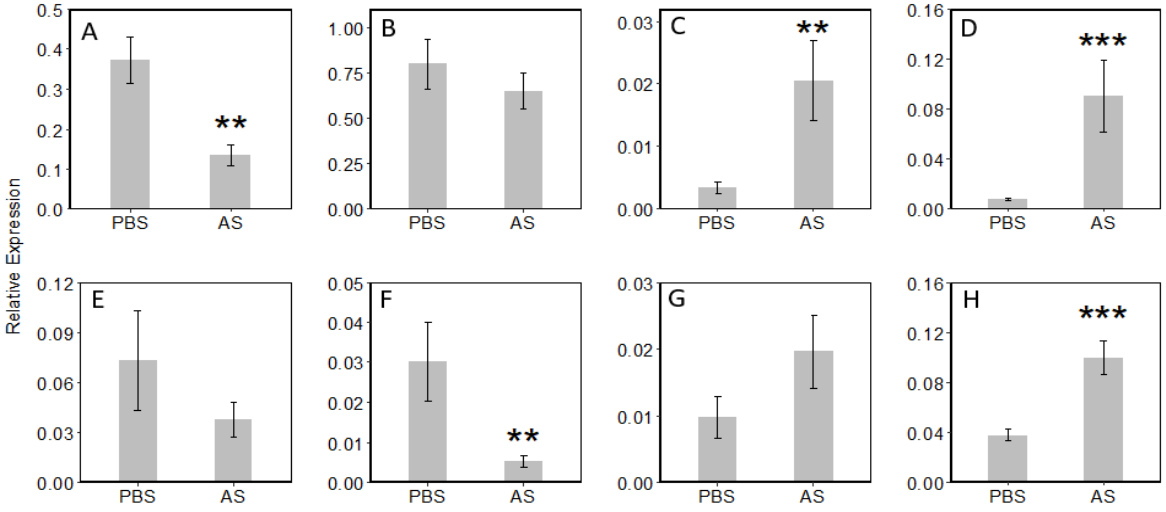
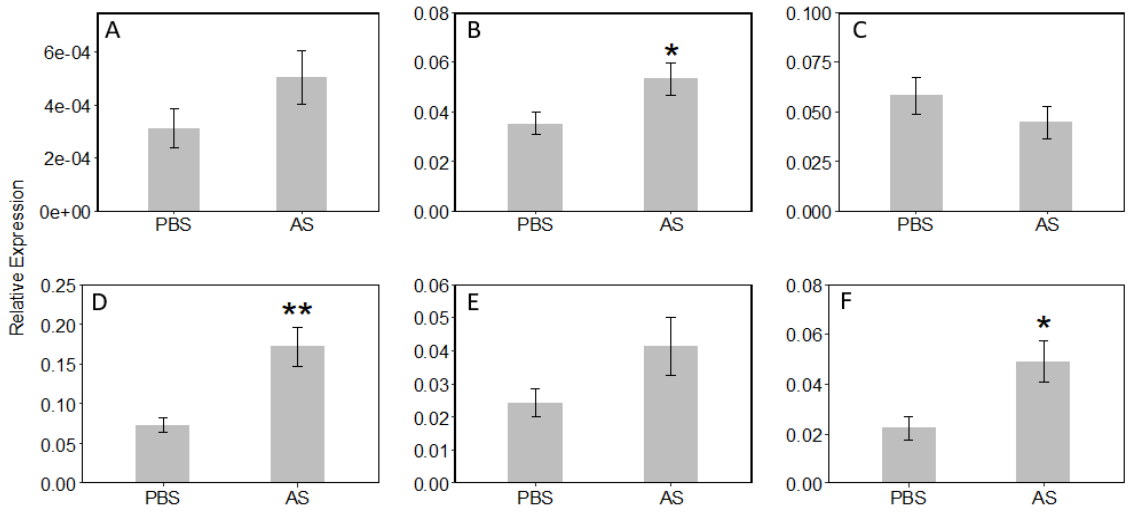


*Figure 8. Relative expression of rainbow trout Arginase paralogues following a bacterial infection. Fish were injected intraperitoneally with either phosphate buffered saline (PBS) or Aeromonas salmonicda (AS). Top row represents gene expression of ARG1a (A), ARG1b (B), ARG2a (C) and ARG2b (D) in liver tissue, expression was normalised to the house keeping genes EF-1α, ACTB and HPRT. Bottom row represents gene expression of ARG1a (E), ARG1b (F), ARG2a (G) and ARG2b (H) in muscle tissue, expression was normalised to the house keeping genes EF-1α, RPS29 and HPRT. Bars represent mean (± SEM), n=10, * = p < 0.05, ** = p < 0.01, *** = p < 0.001*



356

357 *Figure 9. Relative expression of rainbow trout OTC (A), ASS (B) and ASL (C) in liver tissue following a bacterial*
358 *infection. Second row represents relative expression of rainbow trout OTC (D), ASS (E) and ASL (F) in muscle*
359 *tissue. Fish were injected intraperitoneally with either phosphate buffered saline (PBS) or Aeromonas salmonicda*
360 *(AS). For the genes examined in the liver, expression was normalised to housekeeping genes EF-1α, ACTB and*
361 *HPRT. For the bottom panel where muscle tissue was examined, expression was normalised to housekeeping*
362 *genes EF-1α, RPS29 and HPRT. Bars represent mean (± SEM), n=10, * = p < 0.05, ** = p < 0.01, *** = p < 0.001*

363

4. Discussion

Arginine metabolism and the urea cycle are major components of nitrogen metabolism, the inflammatory response and subsequent tissue repair. However, there is a lack of information available in salmonid fish regarding the genes regulating these metabolic pathways. Here we address this knowledge gap by characterising the key enzymes in the urea pathway and by documenting their transcriptional responses to a bacterial infection.

In fish, arginine is often regarded as an essential amino acid required for efficient protein synthesis, in addition to other functional processes influencing health status [5, 36]. For arginine to be synthesised endogenously, carbamoylphosphate, an intermediate molecule in the urea cycle, is combined with ornithine to form citrulline through the action of *OTC* (Fig. 1). In ureotelic mammals and amphibians, carbamoylphosphate synthetase (*CPS*) catalyses the formation of carbamoylphosphate, allowing its use in the urea cycle [37, 38]. In rainbow trout, *CPS* is expressed at early life stages but not in the liver of adults [37] with low levels in muscle [39]. This lack of hepatic *CPS* activity in salmonids could be the reason for an incomplete urea cycle and inability to synthesise arginine endogenously. Some teleosts including the toadfish *Opsanus beta* [40], the catfish *Clarias batrachus* [41], and the lungfish *Protopterus aethiopicus* [42], have detectable *CPS* activity and a functional urea cycle. However, our gene expression data suggests that the urea cycle enzymes are functional in adult rainbow trout and the modulation we observed following AS infection indicates a role during the inflammatory response.

Our phylogenetic analyses revealed that *ODC, SAMdc* and *ARG* have multiple paralogues retained from the salmonid-specific whole genome duplication (ssWGD), which occurred 88-103 Mya [43, 44]. In the case of *ARG,* we observed four salmonid copies, with two paralogues retained for both *ARG1* and *ARG2*, which was also recently shown for Atlantic salmon [20], which are conserved in all vertebrates and presumably the result of an early gene duplication event, perhaps past WGD events in the vertebrate ancestor [45]. However, we did not identify any duplicated paralogues of the key urea cycle genes retained from the teleost-specific WGD event [45]. The duplicated copies that are retained either specialise in function (subfunctionilization) or develop a novel function (neofunctionilization) [46, 47]. Following a WGD event, the resulting duplicated genome eventually only retains a small percentage of duplicated genes, as the redundant genes are inactivated by a process termed gene fractionation [26]. The ssWGD is relatively recent when compared to the teleost specific WGD (~100Mya compared to ~300Mya) and this is evident from the large number of duplicated genes still present in the genome (48% of genes with retained ohnologs) [26].

The genes encoding *OTC, ASS* and *ASL* were found to be present as single copy genes suggesting one duplicated copy was lost in the ancestor of the trout and salmon lineage.

The genes encoding the urea cycle molecules *ARG, OTC, ASS* and *ASL* were expressed in all tissues examined, with *OTC* showing negligible levels in liver; this latter observation may contribute to a low functioning urea cycle in salmonids, and other teleost's [37]. However, genes encoding the other urea cycle enzymes (*ARG, ASS* and *ASL)* were transcribed in the liver, indicating components of the urea cycle may have other metabolic roles beyond nitrogenous waste excretion. The expression of *ASS* and *ASL,* may indicate an efficient conversion of citrulline to arginine in adult trout, but the lack of the carbamoyl substrate is a limiting factor. If the urea cycle is fully functioning, then citrulline could be used to bolster arginine levels, as is found in mammalian species [48, 49]. This idea is also supported by the functioning NOS cycle in salmonids as they are able to produce NO in the innate immune response against pathogens [50]. Citrulline is generated as a by-product of the *iNOS* reaction and could be recycled back into arginine by *ASS* and *ASL.* The arginase genes show an interesting expression profile, *ARG 1a* and *1b* are expressed at high levels in the liver whereas *ARG 2a* and *2b* are virtually absent in liver tissue. For genes encoding polyamine enzymes there is low level of expression in non-stimulated liver and small difference between paralogue expression.

To gain information on the expression of the urea cycle genes/paralogues, we quantified their mRNA expression and transcriptional responses following bacterial infection in muscle and liver. We sampled liver as a key indicator of the acute phase response [51, 52] that shows a well-established response to bacterial infection, while also acting as the main site for amino acid metabolism and the urea cycle. Skeletal muscle was also selected for analysis, due to high transcript levels identified in many of the genes in the tissue distributions (see Results section 3.2.1). Fish were sampled 48h after infection to represent the early immune response before physiological changes due to disease could occur [51]. There were significant increases in liver expression for the polyamine genes *ODC1, 2* and *SAMdc1* and *2* suggesting upregulation of the polyamine pathway and subsequent production of putrescine. This could be related to cellular repair and also potential increased availability of ornithine from increased *ARG2* activity following infection.

Both the *ARG2* paralogues increased in expression following infection, whist *ARG1a* was significantly decreased showing potential subfunctionalisation of the duplicated genes. Recent research has suggested that *ARG1* is involved as a major metaboliser of hepatic arginine whereas *ARG2* may be more involved with the immune response in the form of healing M2 macrophages [19]. During an immune response M2 macrophages demonstrate

elevated levels of arginase activity and also play an important role in the innate immune defence against various pathogens in both a bactericidal and healing sense [18]. The two major types of macrophages, M1 and M2, both depend on the same substrate (arginine) for either healing (M2) or bactericidal activity (M1). The enzymes *iNOS* and arginase have been described as useful markers for M1 and M2 macrophages (respectively) in both mammals [53] and some fish species [54]. As both *iNOS* and arginase compete for arginine they can regulate each other's expression either driving an inflammatory response via the nitric oxide cycle or wound healing from polyamine synthesis by *ODC* [53]. The increased expression of *ARG2* along with *ODC* and *SAMdc* following infection shown in this study suggests that the conversion of arginine into polyamines is taking place for tissue repair. This is in agreement with studies in humans where arginine derived from ornithine also plays a role in tissue remodelling as high levels of arginase can be observed in fibroblasts of patients suffering from pulmonary fibrosis [55]. Further evidence showing the regulatory effect *ARG* and *iNOS* have on each other can be seen in mice when infected with *Helicobacter pylori*, arginase 2 knockout lead to increased M1 macrophage activation [56].

The transcriptional changes seen in the liver from *ARG2* and the polyamine synthesis genes *ODC* and *SAMdc* suggests a signature activation of wound healing M2 macrophages [21]. Although macrophages are virtually present in all tissues [20], we find in muscle, that during inflammation there is a less dramatic response compared to liver. There were no significant changes in the muscle for genes involved in polyamine synthesis suggesting liver is a major source for these molecules. For *ARG* genes there was a similar pattern in muscle and liver indicating conserved regulation between tissues and a conserved inflammatory response. The urea cycle enzymes *(OTC, ASS* and *ASL*) displayed some variation between tissue where *OTC* and *ASL* were significantly upregulated in muscle but unchanged in liver while *ASS* was significantly increased in liver but unchanged in muscle.

5. Conclusion

In summary, the genes encoding the enzymes of the urea cycle and the two rate limiting enzymes in polyamine synthesis have been characterised and their response to infection investigated. Our findings demonstrate that *ARG 1* and *2, SAMdc* and *ODC* genes have retained functional paralogues from the salmonid-specific WGD, with several of the duplicated copies showing different regulation across tissues. The nutritional requirement of arginine in the diet in salmonids is likely to be due to a lack of activity from *CPS* and *OTC* enzymes in adult liver. It is likely that half of the urea cycle is functional and the enzymes responsible for the conversion of citrulline to arginine are active, especially due to the

functioning NO cycle. We also observed significant changes in the urea/polyamine pathways following bacterial challenge, suggesting enhanced recycling and metabolism of arginine for both inflammatory and tissue healing roles following infection.

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References

[1] Krebs, H.A. and Henseleit, K., 1932. *Hoppe-Seyler's Z. physiol. Chem,* 210, pp. 33–66.

[2] Kajimura, M., Croke, S.J., Glover, C.N. and Wood, C.M., 2004. Dogmas and controversies in the handling of nitrogenous wastes: The effect of feeding and fasting on the excretion of ammonia, urea and other nitrogenous waste products in rainbow trout. *Journal of Experimental Biology*, 207(12), pp. 1993-2002.

[3] Morris Jr., S.M., 2002. Regulation of enzymes of the urea cycle and arginine metabolism. *Annual Review of Nutrition*, 22 (2002), pp. 87-105

[4] Li, P., Mai, K., Trushenski, J. and Wu, G., 2009. New developments in fish amino acid nutrition: Towards functional and environmentally oriented aquafeeds. *Amino acids*, 37(1), pp. 43-53.

[5] Mommsen, T.P., Moon, T.W. and Plisetskaya, E.M., 2001. Effects of arginine on pancreatic hormones and hepatic metabolism in rainbow trout. *Physiological and Biochemical Zoology*, 74(5), pp. 668-678.

[6] Ozer, L., Elgun, S., Ozdemir, B., Pervane, B. and Ozmeric, N., 2011. Arginine-nitric oxide-polyamine metabolism in periodontal disease. *Journal of Periodontology*, 82(2), pp. 320-328.

[7] Balaña-Fouce, R., Calvo-Álvarez, E., Álvarez-Velilla, R., Prada, C.F., Pérez-Pertejo, Y. and Reguera, R.M., 2012. Role of *trypanosomatid's* arginase in polyamine biosynthesis and pathogenesis*. Molecular and biochemical parasitology*, 181(2), pp. 85-93.

[8] Wu, G. And Morris Jr., S.M., 1998. Arginine metabolism: Nitric oxide and beyond. *Biochemical Journal*, 336(1), pp. 1-17.

[9] Uehara, E.U., Shida, B.S. and De Brito, C.A., 2015. Role of nitric oxide in immune responses against viruses: beyond microbicidal activity. *Inflammation Research,* 64(11), pp. 845-852.

[10] Igarashi, K. and Kashiwagi, K., 2010. Modulation of cellular function by polyamines. *International Journal of Biochemistry and Cell Biology*, 42(1), pp. 39-51.

[11] Williams, K., 1997. Modulation and block of ion channels: A new biology of polyamines. *Cellular signalling*, 9(1), pp. 1-13.

[12] Giordano, E., Flamigni, F., Guarnieri, C., Muscari, C., Pignatti, C., Stefanelli, C., Tantini, B. and Caldarera, C.M., 2010. Polyamines in cardiac physiology and disease. O*pen Heart Failure Journal, 3(SPEC. ISS.1)*, pp. 25-30.

[13] Messina, L., Arcidiacono, A., Spampinato, G., Malaguarnera, L., Berton, G., Kaczmarek, L. and Messina, A., 1990. Accumulation of ornithine decarboxylase

513 mRNA accompanies activation of human and mouse monocytes/macrophages.
514 *FEBS letters*, 268(1), pp. 32-34.
515 [14] Hussain, T., Tan, B., Ren, W., Rahu, N., Dad, R., Kalhoro, D.H. and Yin, Y., 2017.
516 Polyamines: therapeutic perspectives in oxidative stress and inflammatory diseases.
517 *Amino acids,* 49(9), pp. 1457-1468.
518 [15] Nishimura, K., Murozumi, K., Shirahata, A., Park, M.H., Kashiwagi, K. and Igarashi,
519 K., 2005. Independent roles of eIF5A and polyamines in cell proliferation.
520 *Biochemical Journal*, 385(3), pp. 779-785.
521 [16] Michael, A.J., 2016. Biosynthesis of polyamines and polyamine-containing
522 molecules. *Biochemical Journal*, 473(15), pp. 2315-2329.
523 [17] Moinard, C., Cynober, L. and de Bandt, J.P., 2005. Polyamines: Metabolism and
524 implications in human diseases. *Clinical Nutrition*, 24(2), pp. 184-197.
525 [18] Benedicenti, O., Wang, T., Wangkahart, E., Milne, D.J., Holland, J.W., Collins, C.
526 and Secombes, C.J., 2017. Characterisation of arginase paralogues in salmonids
527 and their modulation by immune stimulation/ infection. *Fish and Shellfish*
528 *Immunology*, 61, pp. 138-151.
529 [19] Wiegertjes, G.F., Wentzel, A.S., Spaink, H.P., Elks, P.M. and Fink, I.R., 2016.
530 Polarization of immune responses in fish: The 'macrophages first' point of view.
531 *Molecular immunology*, 69, pp. 146-156.
532 [20] Mosser, D.M. and Edwards, J.P., 2008. Exploring the full spectrum of macrophage
533 activation. *Nature Reviews Immunology*, 8(12), pp. 958-969.
534 [21] Hardbower, D.M., Asim, M., Luis, P.B., Singh, K., Barry, D.P., Yang, C., Steeves,
535 M.A., Cleveland, J.L., Schneider, C., Piazuelo, M.B., Gobert, A.P. and Wilson, K.T.,
536 2017. Ornithine decarboxylase regulates M1 macrophage activation and mucosal
537 inflammation via histone modifications. *Proceedings of the National Academy of*
538 *Sciences of the United States of America,* 114(5), pp. E751-E760.
539 [22] Zheng, L., Pan, Y., Feng, Y., Cui, L. and Cao, Y., 2015. L-Arginine supplementation
540 in mice enhances NO production in spleen cells and inhibits *Plasmodium yoelii*
541 transmission in mosquitoes. *Parasites and Vectors*, 8(1).
542 [23] Oehme, M., Grammes, F., Takle, H., Zambonino-Infante, J.-., Refstie, S.,
543 Thomassen, M.S., Rørvik, K. and Terjesen, B.F., 2010. Dietary supplementation of
544 glutamate and arginine to Atlantic salmon (*Salmo salar L*.) increases growth during
545 the first autumn in sea. *Aquaculture*, 310(1-2), pp. 156-163.
546 [24] Chen, G., Liu, Y., Jiang, J., Jiang, W., Kuang, S., Tang, L., Tang, W., Zhang, Y.-.,
547 Zhou, X. and Feng, L., 2015. Effect of dietary arginine on the immune response and
548 gene expression in head kidney and spleen following infection of Jian carp with
549 *Aeromonas hydrophila*. *Fish and Shellfish Immunology*, 44(1), pp. 195-202.
550 [25] Pereira, R.T., Rosa, P.V. and Gatlin, D.M., 2017. Glutamine and arginine in diets for
551 Nile tilapia: Effects on growth, innate immune responses, plasma amino acid profiles
552 and whole-body composition. *Aquaculture*, 473, pp. 135-144.
553 [26] Berthelot, C., Brunet, F., Chalopin, D., Juanchich, A., Bernard, M., Noël, B., Bento,
554 P., Da Silva, C., Labadie, K., Alberti, A., Aury, J.-., Louis, A., Dehais, P., Bardou, P.,
555 Montfort, J., Klopp, C., Cabau, C., Gaspin, C., Thorgaard, G.H., Boussaha, M.,
556 Quillet, E., Guyomard, R., Galiana, D., Bobe, J., Volff, J.-., Genêt, C., Wincker, P.,
557 Jaillon, O., Crollius, H.R. and Guiguen, Y., 2014. The rainbow trout genome
558 provides novel insights into evolution after whole-genome duplication in vertebrates.
559 *Nature Communications*, 5.
560 [27] Hubbard, T., Barker, D., Birney, E., Cameron, G., Chen, Y., Clark, L., Cox, T., Cuff,
561 J., Curwen, V., Down, T., Durbin, R., Eyras, E., Gilbert, J., Hammond, M.,
562 Huminiecki, L., Kasprzyk, A., Lehvaslaiho, H., Lijnzaad, P., Melsopp, C., Mongin, E.,
563 Pettett, R., Pocock, M., Potter, S., Rust, A., Schmidt, E., Searle, S., Slater, G.,

564 Smith, J., Spooner, W., Stabenau, A., Stalker, J., Stupka, E., Ureta-Vidal, A.,
565 Vastrik, I. and Clamp, M., 2002. The Ensembl genome database project. *Nucleic*
566 *acids research*, 30(1), pp. 38-41.
567 [28] Campanella, J.J., Bitincka, L. and Smalley, J., 2003. MatGAT: An application that
568 generates similarity/identity matrices using protein or DNA sequences. *BMC*
569 *Bioinformatics*, 4.
570 [29] Kumar, S., Stecher, G. and Tamura, K., 2016. MEGA7: Molecular Evolutionary
571 Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular biology and evolution*,
572 33(7), pp. 1870-1874.
573 [30] Mcwilliam, H., Li, W., Uludag, M., Squizzato, S., Park, Y.M., Buso, N., Cowley, A.P.
574 and Lopez, R., 2013. Analysis Tool Web Services from the EMBL-EBI. *Nucleic acids*
575 *research*, 41(Web Server issue), pp. W597-600.
576 [31] KIBBE, W.A., 2007. OligoCalc: An online oligonucleotide properties calculator.
577 *Nucleic acids research,* 35(SUPPL.2), pp. W43-W46.
578 [32] Alzaid, A., Castro, R., Wang, T., Secombes, C.J., Boudinot, P., Macqueen, D.J. and
579 Martin, S.A.M., 2016. Cross talk between growth and immunity: Coupling of the igf
580 axis to conserved cytokine pathways in rainbow trout. *Endocrinology*, 157(5), pp.
581 1942-1955.
582 [33] Micallef, G., Cash, P., Fernandes, J.M.O., Rajan, B., Tinsley, J.W., Bickerdike, R.,
583 Martin, S.A.M. and Bowman, A.S., 2017. Dietary yeast cell wall extract alters the
584 proteome of the skin mucous barrier in Atlantic salmon (*Salmo salar*): Increased
585 abundance and expression of a calreticulin-like protein. *PLoS ONE*, 12(1).
586 [34] Heidari, Z., Bickerdike, R., Tinsley, J., Zou, J., Wang, T., Chen, T. and Martin,
587 S.A.M., 2015. Regulatory factors controlling muscle mass: Competition between
588 innate immune function and anabolic signals in regulation of atrogin-1 in Atlantic
589 salmon. *Molecular immunology*, 67(2), pp. 341-349.
590 [35] Ruijter, J.M., Ramakers, C., Hoogaars, W.M.H., Karlen, Y., Bakker, O., Van Den
591 Hoff, M.J.B. and Moorman, A.F.M., 2009. Amplification efficiency: Linking baseline
592 and bias in the analysis of quantitative PCR data. *Nucleic acids research*, 37(6).
593 [36] Berge, G.E., Lied, E. and Sveier, H., 1997. Nutrition of Atlantic salmon (*Salmo*
594 *salar*): The requirement and metabolism of arginine. *Comparative Biochemistry and*
595 *Physiology - A Physiology*, 117(4), pp. 501-509.
596 [37] Korte, J.J., Salo, W.L., Cabrera, V.M., Wright, P.A., Felskie, A.K. and Anderson,
597 P.M., 1997. Expression of carbamoyl-phosphate synthetase III mRNA during the
598 early stages of development and in muscle of adult rainbow trout (*Oncorhynchus*
599 *mykiss*). *Journal of Biological Chemistry*, 272(10), pp. 6270-6277.
600 [38] Thoden, J.B., Miran, S.G., Phillips, J.C., Howard, A.J., Raushel, F.M. and Holden,
601 H.M., 1998. Carbamoyl phosphate synthetase: Caught in the act of glutamine
602 hydrolysis. *Biochemistry,* 37(25), pp. 8825-8831.
603 [39] Todgham, A.E., Anderson, P.M. And Wright, P.A., 2001. Effects of exercise on
604 nitrogen excretion, carbamoyl phosphate synthetase III activity and related urea
605 cycle enzymes in muscle and liver tissues of juvenile rainbow trout (*Oncorhynchus*
606 *mykiss*). *Comparative Biochemistry and Physiology - A Molecular and Integrative*
607 *Physiology*, 129(2-3), pp. 527-539.
608 [40] Laberge, T., Walsh, P.J. and Mcdonald, M.D., 2009. Effects of crowding on
609 ornithine-urea cycle enzyme mRNA expression and activity in gulf toadfish
610 (*Opsanus beta*). *Journal of Experimental Biology*, 212(15), pp. 2394-2402.
611 [41] Saha, N., Datta, S., Kharbuli, Z.Y., Biswas, K. and Bhattacharjee, A., 2007. Air-
612 breathing catfish, *Clarias batrachus* upregulates glutamine synthetase and carbamyl
613 phosphate synthetase III during exposure to high external ammonia. *Comparative*

Biochemistry and Physiology - B Biochemistry and Molecular Biology, 147(3), pp. 520-530.

[42] Ai, M.L., Kum, C.H., Lee, S.M.L., Wai, P.W., Shit, F.C. and Yuen, K.I., 2005. Ornithine-urea cycle and urea synthesis in African lungfishes, *Protopterus aethiopicus* and *Protopterus annectens*, exposed to terrestrial conditions for six days. *Journal of Experimental Zoology Part A: Comparative Experimental Biology*, 303(5), pp. 354-365.

[43] Macqueen, D.J. and Johnston, I.A., 2014. A well-constrained estimate for the timing of the salmonid whole genome duplication reveals major decoupling from species diversification. *Proceedings of the Royal Society B: Biological Sciences,* 281(1778),.

[44] Lien, S., Koop, B.F., Sandve, S.R., Miller, J.R., Kent, M.P., Nome, T., Hvidsten, T.R., Leong, J.S., Minkley, D.R., Zimin, A., Grammes, F., Grove, H., Gjuvsland, A., Walenz, B., Hermansen, R.A., Von Schalburg, K., Rondeau, E.B., Di Genova, A., Samy, J.K.A., Olav Vik, J., Vigeland, M.D., Caler, L., Grimholt, U., Jentoft, S., Inge Våge, D., De Jong, P., Moen, T., Baranski, M., Palti, Y., Smith, D.R., Yorke, J.A., Nederbragt, A.J., Tooming-Klunderud, A., Jakobsen, K.S., Jiang, X., Fan, D., Hu, Y., Liberles, D.A., Vidal, R., Iturra, P., Jones, S.J.M., Jonassen, I., Maass, A., Omholt, S.W. and Davidson, W.S., 2016. The Atlantic salmon genome provides insights into rediploidization. *Nature*, 533, pp. 200-205.

[45] Volff, J., 2005. Genome evolution and biodiversity in teleost fish. *Heredity*, 94(3), pp. 280-294.

[46] Pasquier, J., Cabau, C., Nguyen, T., Jouanno, E., Severac, D., Braasch, I., Journot, L., Pontarotti, P., Klopp, C., Postlethwait, J.H., Guiguen, Y. and Bobe, J., 2016. Gene evolution and gene expression after whole genome duplication in fish: The PhyloFish database. *BMC Genomics*, 17(1).

[47] Glasauer, S.M.K. and Neuhauss, S.C.F., 2014. Whole-genome duplication in teleost fishes and its evolutionary consequences. *Molecular Genetics and Genomics*, 289(6), pp. 1045-1060.

[48] Ham, D.J., Gleeson, B.G., Chee, A., Baum, D.M., Caldow, M.K., Lynch, G.S. and Koopman, R., 2015. L-Citrulline protects skeletal muscle cells from cachectic stimuli through an iNOS-Dependent Mechanism. *PLoS ONE*, 10(10).

[49] Wijnands, K.A.P., Vink, H., Briedé, J.J., Van Faassen, E.E., Lamers, W.H., Buurman, W.A. and Poeze, M., 2012. Citrulline a more suitable substrate than arginine to restore no production and the microcirculation during endotoxemia. *PLoS ONE*, 7(5).

[50] Estévez, R.A., Mostazo, M.G.C., Rodriguez, E., Espinoza, J.C., Kuznar, J., Jónsson, Z.O., Guðmundsson, G.H. and Maier, V.H., 2018. Inducers of salmon innate immunity: An in vitro and in vivo approach. *Fish and Shellfish Immunology*, 72, pp. 247-258

[51] Causey, D.R., Pohl, M.A.N., Stead, D.A., Martin, S.A.M., Secombes, C.J. and Macqueen, D.J., 2018. High-throughput proteomic profiling of the fish liver following bacterial infection. *BMC Genomics*, 19(1),

[52] Gerwick, L., Corley-Smith, G. and Bayne, C.J., 2007. Gene transcript changes in individual rainbow trout livers following an inflammatory stimulus*. Fish and Shellfish Immunology*, 22(3), pp. 157-171.

[53] Rath, M., Müller, I., Kropf, P., Closs, E.I. and Munder, M., 2014. Metabolism via arginase or nitric oxide synthase: Two competing arginine pathways in macrophages*. Frontiers in Immunology,* 5, pp. 532.

[54] Forlenza, M., Fink, I.R., Raes, G. and Wiegertjes, G.F., 2011. Heterogeneity of macrophage activation in fish. *Developmental and comparative immunology*, 35(12), pp. 1246-1255.

[55] Kitowska, K., Zakrzewicz, D., Königshoff, M., Chrobak, I., Grimminger, F., Seeger, W., Bulau, P. and Eickelberg, O., 2008. Functional role and species-specific contribution of arginases in pulmonary fibrosis. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 294(1), pp. L34-L45

[56] Hardbower, D.M., Asim, M., Murray-Stewart, T., Casero, R.A., Verriere, T., Lewis, N.D., Chaturvedi, R., Piazuelo, M.B. and Wilson, K.T., 2016. Arginase 2 deletion leads to enhanced M1 macrophage activation and upregulated polyamine metabolism in response to *Helicobacter pylori* infection. *Amino acids*, 48(10), pp. 2375-2388.